Introduction to Proteomics
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Lecture – 33
iTRAQ: In vitro labeling

Welcome to the proteomics course, today we will talk about quantitative proteomics and discuss about iTRAQ and TMT techniques. The quantitative proteomics aims to answer various questions including the identification of biomarkers for very reasons and target identification for early diagnostic intervention. Various techniques or strategies have been developed for quantitative proteomics including the gel based approaches such as dyes, which we have discussed in the last module.

And mass spectrometry based measurements, in last class we talked about ICAT, which was first generation MS based quantitative approach for quantification of relative levels of proteins in various control and treatment samples. The ICAT reagent, as we discussed was consists of 3 components; thiol functional groups, a linker group and a biotin moiety, two samples treated with ICAT reagents light or heavy isotope D0 or D8 were selectively alkylated for cysteine residues.

These samples were combined peptic digested affinity purified based on the evidence and then analyzed by the LC/MS based approach, so the ion abundance ratio of light and heavy isotope labeled peptides could provide the relative abundance of these proteins. So, ICAT was the first quantitative MS based approach which was initiated but because of the only 2 samples could be analyzed from this approach.

New approaches came considering the sample multiplexing because one limitation of ICAT was that only 2 labels are available therefore, more samples cannot be compared in a given experiment. So, there is a need to compared large number of treatments that led to development of 2 or 4 plex isotope coded protein label, iCPL 4 or 8 plex isobaric tagging for relative an absolute quantitation iTRAQ and 2 or 6 plex, tandem mass tag TMT labeled based techniques.

So, today we will continue our discussion on iTRAQ and TMT techniques which can compare up to 8 or 6 samples in a single analysis, so these techniques can increase the throughput. So, let

us talk about quantitative proteomics and different in vitro labeling methods, then vitro labeling methods rely on use of labeling reactions at a specific site in proteins or peptides based on various labeling chemistry different type of strategies have been developed to introduce isotopes at either protein or peptide level.

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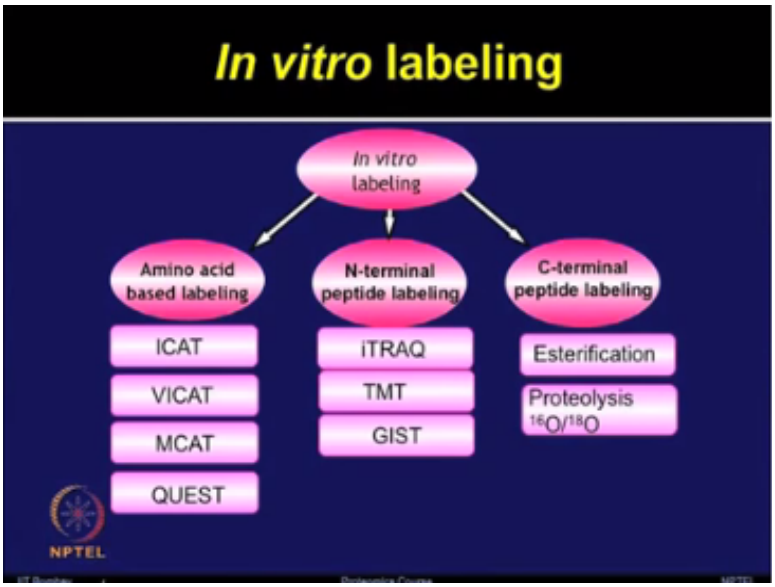

And these strategies will discuss in the next slide, so in vitro labeling can be 3 different types; amino acid based labeling and terminal peptide labelling, C terminal peptide labelling. In the amino acid based labeling such as ICAT or isotope coded affinity tag, there are other amino acid based labeling methods as well such as VICAT or visible isotope coded affinity tag, MCAT or mass coded abundance tagging, QUEST or quantitation using enhanced signal tags.

Then there are different type of end terminal peptide labeling methods such as iTRAQ and TMT which we are discussing today in detail. iTRAQ also known as isobaric tagging for relative and absolute quantitation, TMT's are tandem mass tags and GIST, global internal standard technology. Then there are C terminal based peptide labeling methods such as esterification and proteolysis using 16 or 18 oxygen using different light and heavy form of oxygen.

So, there are varieties of in vitro labeling based approaches available and depending upon your quantitative application, there is possibility of using different type of dyeing strategies. Further discuss that mass spectrometry has played a very major role in proteomics and now it is becoming a very essential tool to study the complex biological system in various diseases.

iTRAQ is a mass spec based technique for relative and absolute quantitation of proteins present in up to 4 samples or up to 8 samples depending upon the type of iTRAQ tags.

And these labels can be provided in the proteins were there N terminal, the iTRAQ labels from the applied based system and tandem mass tax labels are available from the thermo efficient, they are currently the only commercially available tagging technologies, where quantification can be carried out in the MS/MS mode. The iTRAQ labels available from the applied bio systems and tender mass tags available from the thermo efficient are currently the only tagging technologies which are commercially available, where quantitation is carried out in MS/MS mode.

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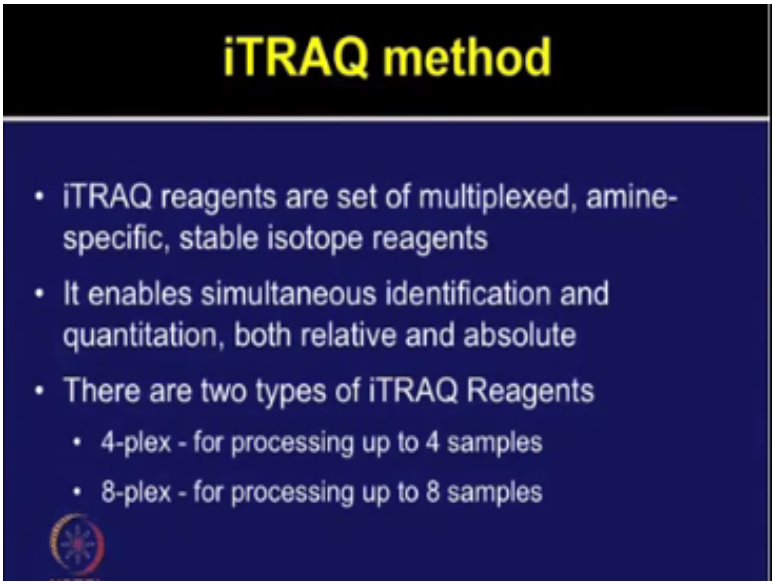

The iTRAQ technique was first time described by Ross et al in 2004, I have given you reference in the bottom in my slide and it was subsequently commercialized by the applied bio systems. So, these iTRAQ reagents are set of multiplexed, amine specific, stable isotope reagents. It enables simultaneous identification and quantitation both relative and absolute. There are two different type of iTRAQ reagents currently available.

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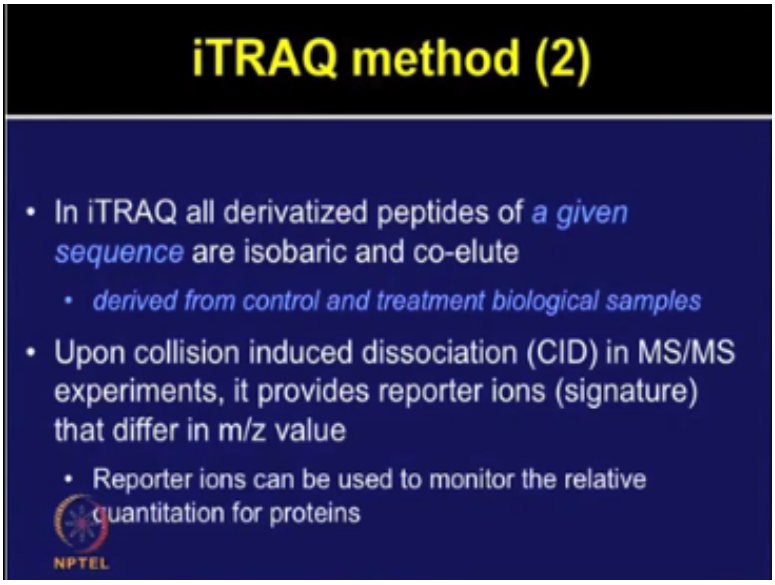

4 plex for processing up to 4 samples and 8 plex for analysis of up to 8 samples. In iTRAQ method, the derivatized peptides of a given sequence, they are isobaric and they are co-eluted because they are derived from the control and treatment biological samples. So, in the mass spec upon collision induced dissociation during the MS/MS experiments, it provides a reporter ion, a signature ion for which differs in mass and charge ratio.

So, the reporter ions are used to track the quantitation and can be used to monitor the relative quantitation for proteins. Now, discuss about the iTRAQ reagent, there are a set of 4 isobaric amine specific labeling regions 114, 115, 116 or 117. The iTRAQ reagent consists of a reporter group, a balancer group and a peptide reactive group PRG. The protein reactive group label the and terminus of all peptides as well as the free amine group of lysine side chains.

The neutral balance portion and reporter group they provide total mass of 145, so this method can allow the multiplexing of up to 4 or 8 different samples in a single LC MS/MS experiment. The different distribution of isotopes between the reporter and balanced group makes the label isobaric and it enables the detection upon fragmentation and release off and the release in mass spec.

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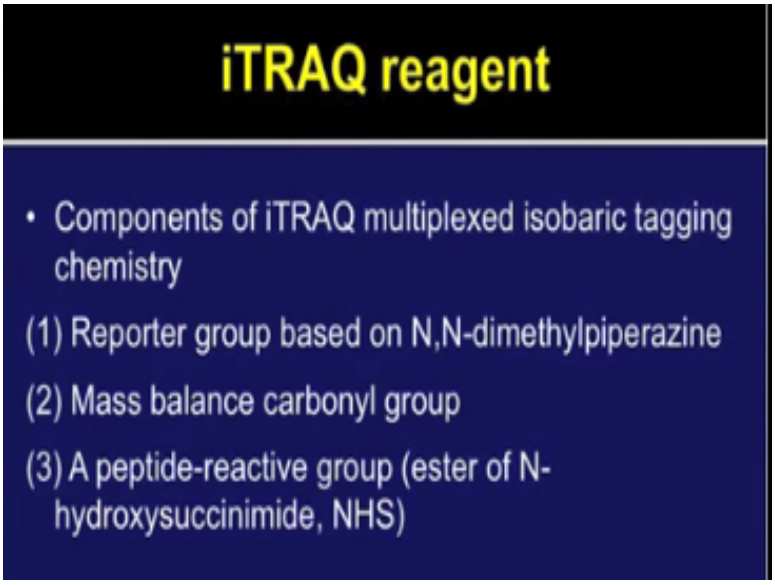

So, as I briefly talked there are 3 major components of the iTRAQ reagent; one is the reporter group which is based on the N-N dimethylpiperazine or DMP, second is a mass balance carbonyl group and third, a peptide reactive group which is an ester of N hydroxysuccinimide NHS.

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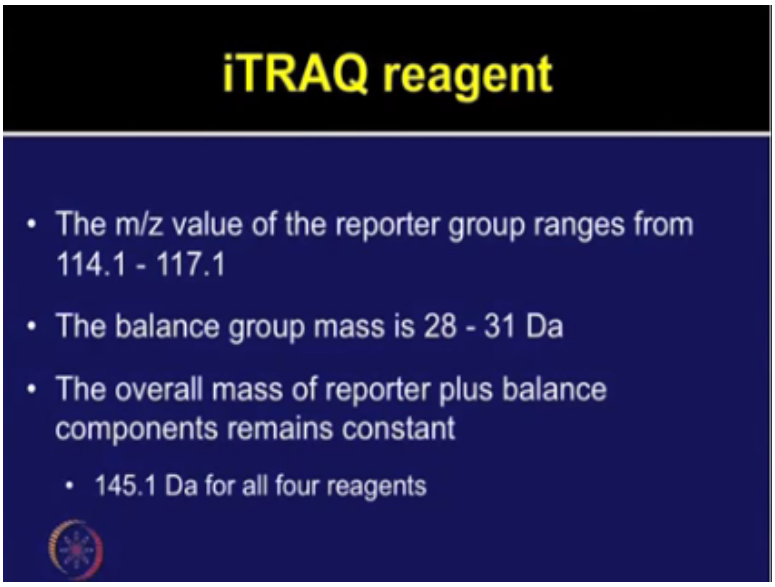

So, the iTRAQ reagent, the master charge value of these reporter groups, they range from 114.1 to 117.1, if you are using a 4 plex reagent. The balance group of masses 28 to 31 Dalton, the overall mass of reporter plus balanced components remains constant, so 145.1 Dalton will remain for all the 4 reagents.

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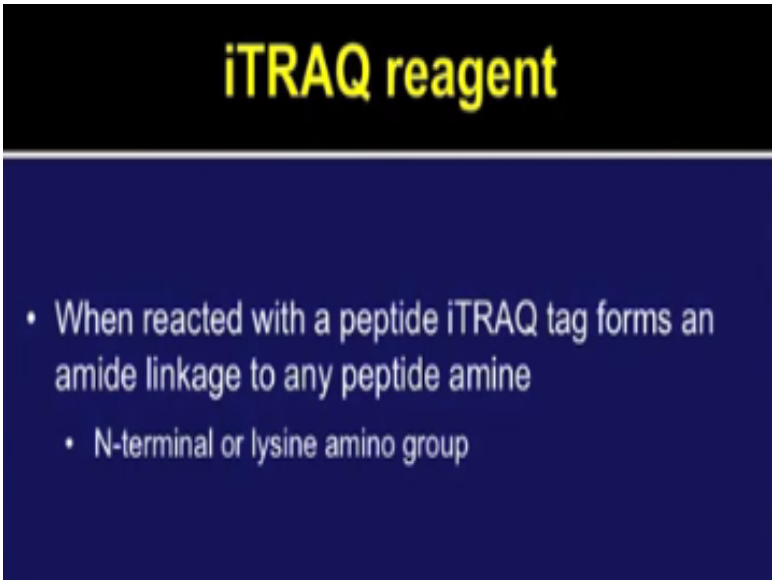

So, when reacted with a peptide iTRAQ tag forms an amide linkage to any peptide amine and terminal or lysine amino group.

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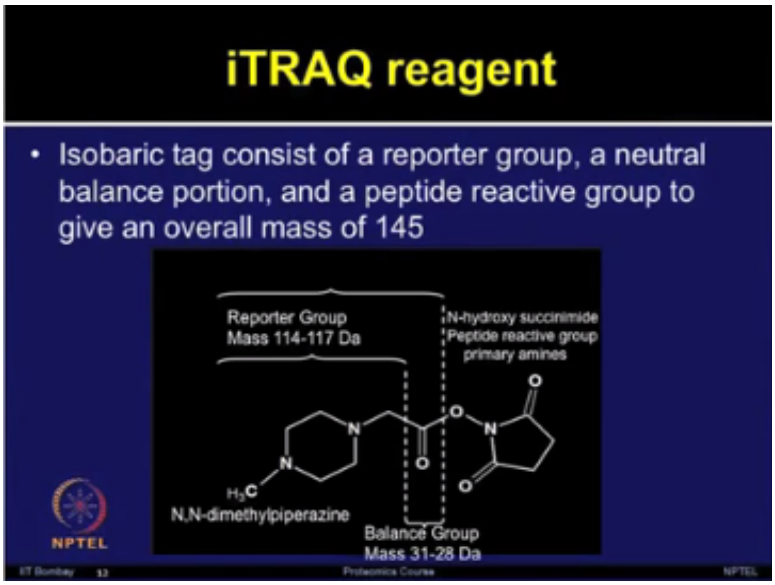

Now, this is a structure of the iTRAQ reagent, where you can see that it consists of a reporter group, a neutral balance portion and a peptide reactive group, an overall mass will remain 145.

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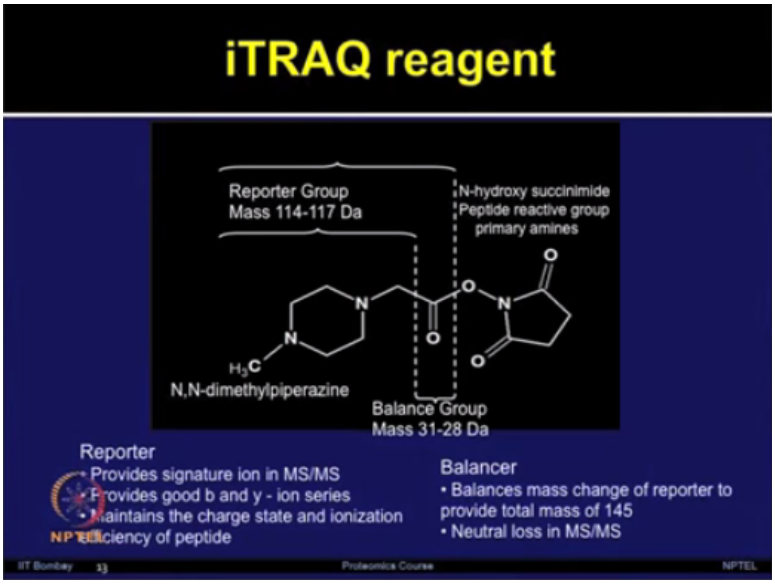

The reporter group provides signature ions in MS/MS mode, it provides good b and y ion series and it maintains the charge state and ionization efficiency of peptides whereas a balancer group it balances the mass change of reporter to provide total mass of 145, neutral loss in MS/MS mode.

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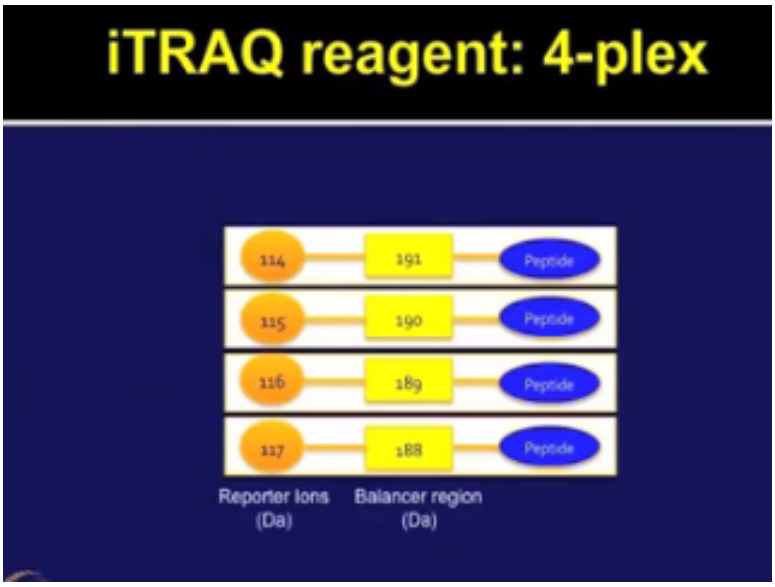

The iTRAQ level is an isobaric tagging compound consisting of a reporter ion series. Here, I have shown you 4 plex, iTRAQ reagent with 114 to 117 Dalton, a balancer region 181 to 188 Dalton, so that the total mass of isobaric tag is 305.

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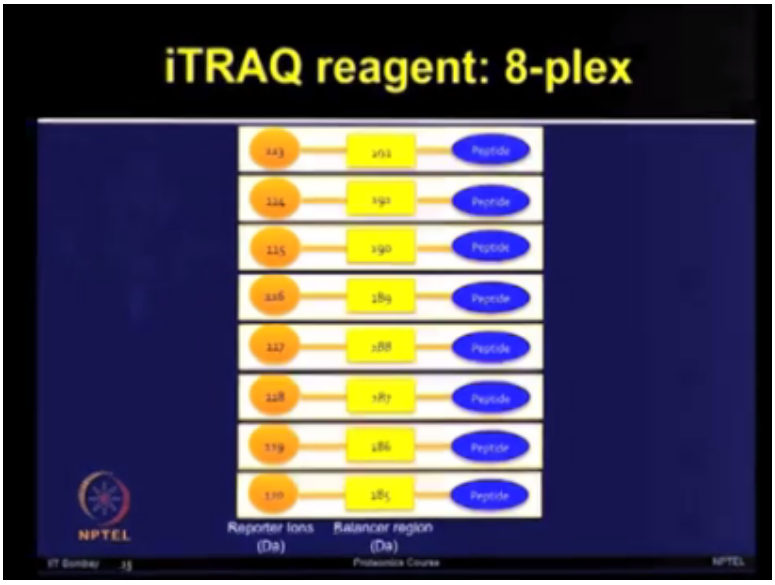

Now, let us look at the structure of iTRAQ reagent 8 plex. In 8 plex in reporter ions, the variable masses from 113 to 120 Dalton and the balancer reagent is from 185 to 192 Dalton, so that the total mass of isobaric tag remains 305. We will not talk about how to perform an iTRAQ experiment. So, in iTARQ experiment, the control and treated protein samples they are first reduced, alkylated and digested with trypsin.

The digested samples are reacted with different0 iTARQ reagents 4 plex or 8 plex, 2 samples are then combined in fact control and 3 treatments or it can be 4 different type of time core samples, so 4 samples can be combined and analyzed by the LC MS/MS, so as per the guidelines provided by the applied Bio systems, the protein samples can be prepared and now we will discuss that step by step in the next few slides.

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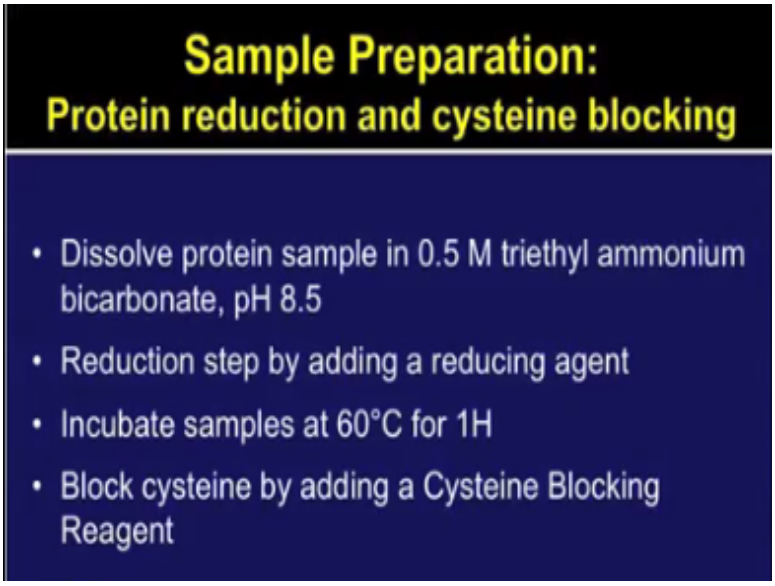

So, the sample preparation, first part is protein reduction and blocking or cystines. So, first dissolve the protein sample in 0.5 molar tri ethyl ammonium bicarbonate at pH 8.5 then perform a reduction step by adding a reducing agent, incubate the samples at 60 degrees for an hour. After that, add a cysteine blocking reagent, so that cysteine residues can be blocked.

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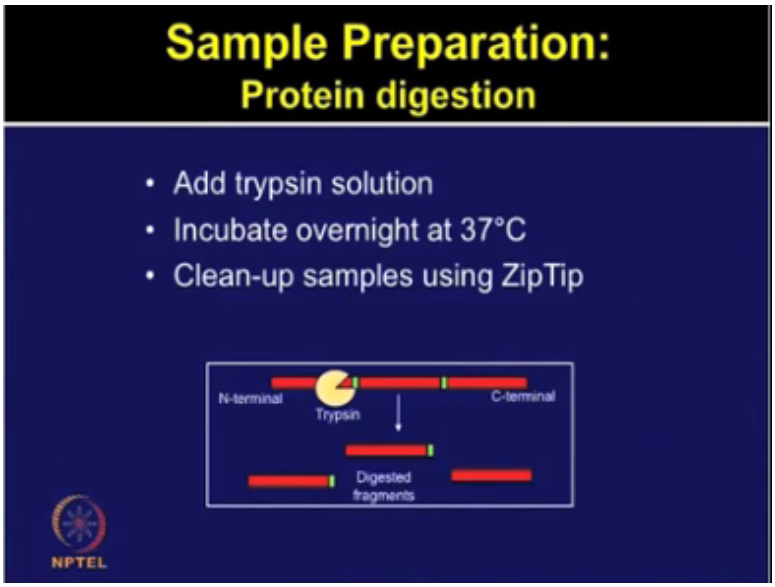

Once reduction and cysteine blocking is done, then protein digestion can be performed, so add trypsin solution incubate overnight at 37 degrees, so that these proteins can be digested into the peptides, you may have discussed this thing briefly in the previous module when we talked about in gel digestion, for doing the gel based proteomics, similar type of concepts are applied here but this one is in solution digestion.

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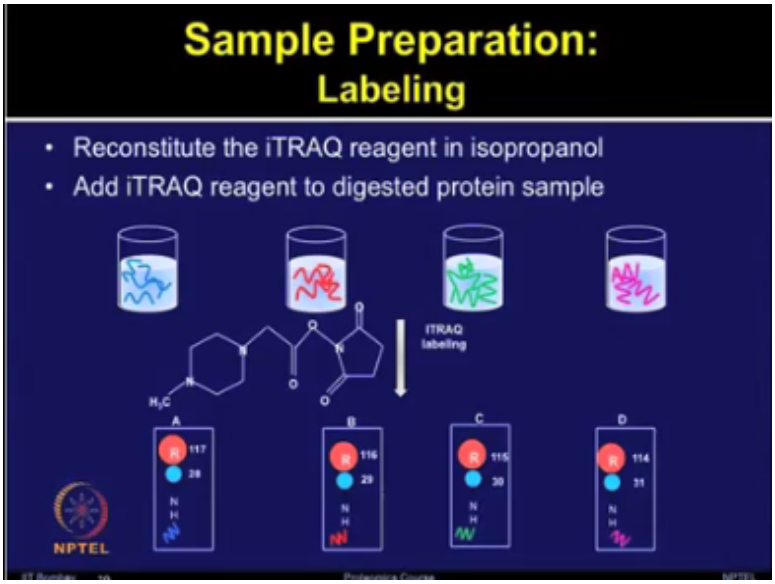

Once you have done the overnight digestion, then a clean-up step can be performed by using zip tip, so that some salts and contaminants can be removed. Now, next step is labelling, adding these iTRAQ reagents to the 4 different samples. So, first reconstitute the iTRAQ reagent in isopropanol, add iTRAQ reagent to the digested protein samples, if you have 4 samples, you can use with the 4 plex iTRAQ reagents; 114, 115, 116 and 117 as I have shown in this PPT.

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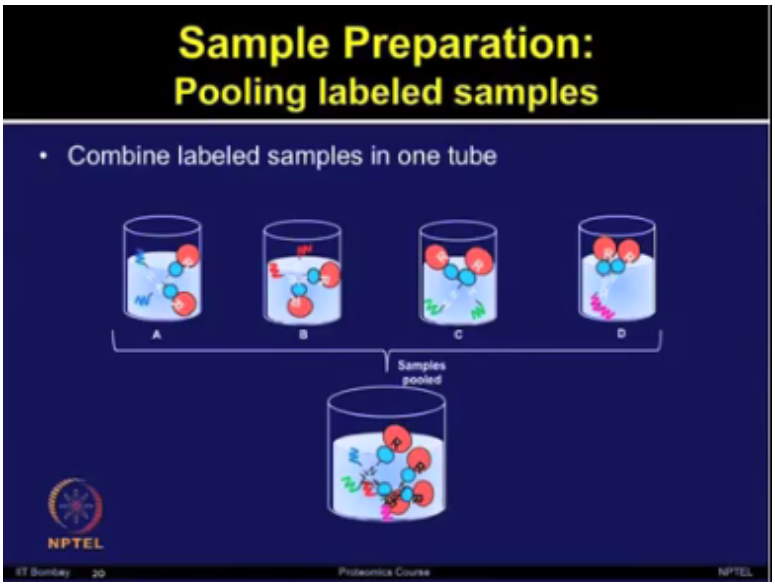

Now, combine these labeled samples in one tube, so that later on sample processing will not have any manual artefact, so the labeling is performed separately but after that, all the label samples are pooled in one common tube.

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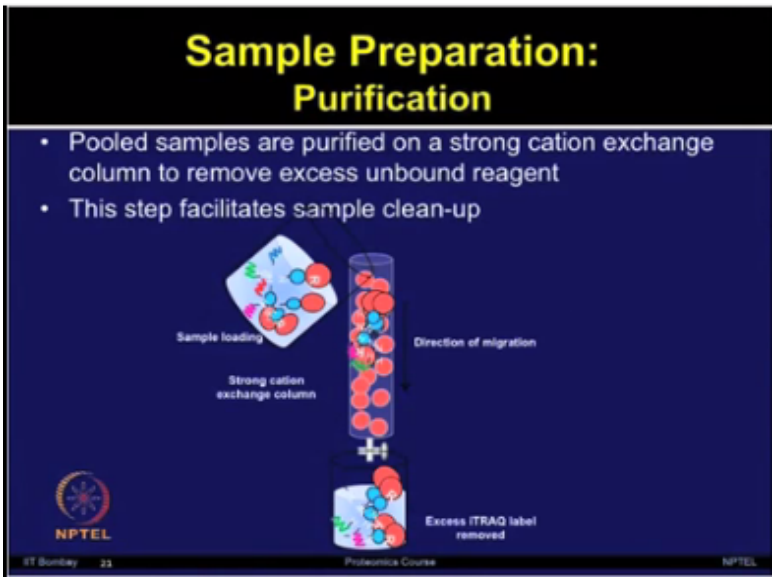

Now, these pooled samples can be further purified on a strong cation exchange column, so that their excess unbound reagents can be removed. This is an optional step but this does help in

proper cleaning and getting the better signal. So, this step facilitates sample clean-up and this is also not essential but recommended to perform the system.

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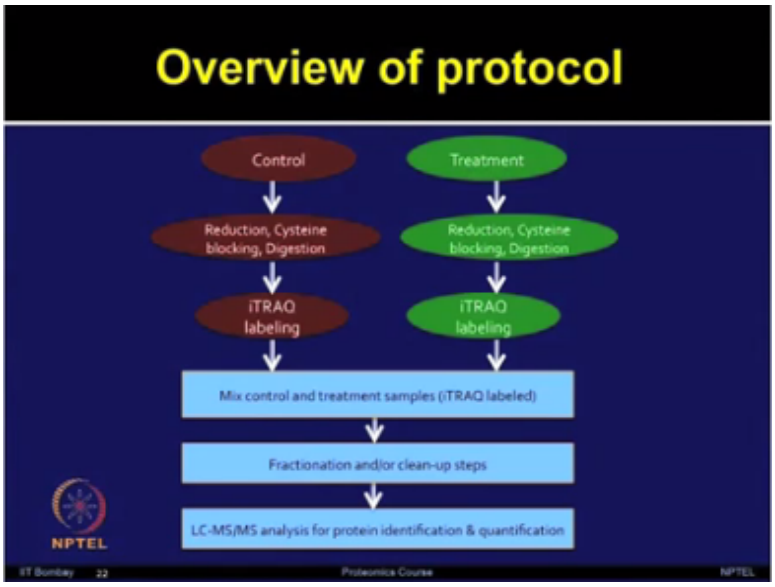

So, let us have a look on the overview of this protocol, so we have control and treatment populations each of those samples were first reduced cysteine blocked and digested after that, iTRAQ labeling was performed for each sample then control and treatment populations were all mixed together with the iTRAQ labels, fractionation and or clean-up steps were performed and then LC MS/MS analysis can be performed for the protein identification and protein quantification.

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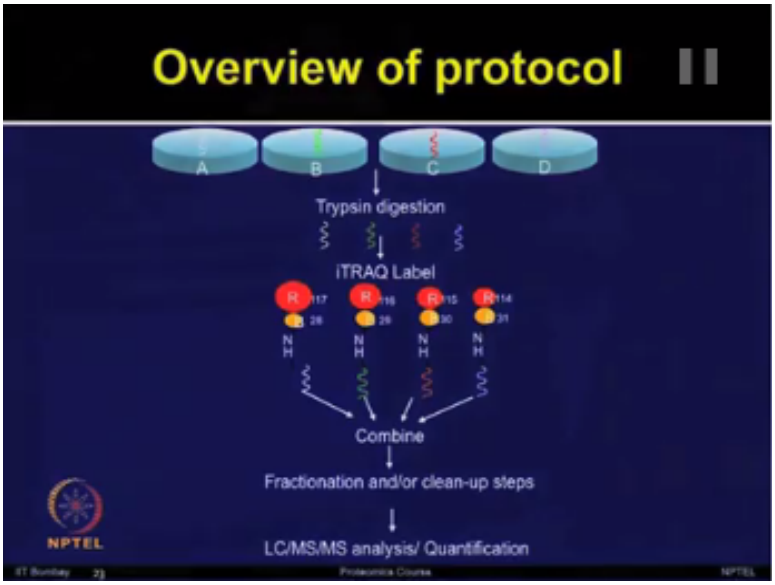

Now, I think this image it will be more clear to you, the overall steps you have 4 different populations; A, B, C and D. Each population is reduced cysteine blocked trypsin digested and

then further labeled with the 4 different type of iTRAQ label, combine fractionated and further LC/MS analysis can be performed and quantification can be obtained. Once the sample preparation is done, then further mass spectrometry analysis can be start.

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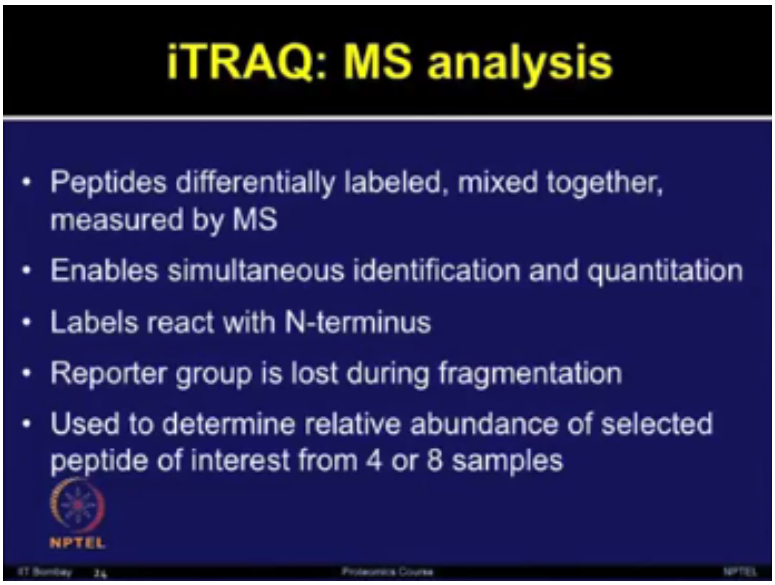

The peptides which are differentially labelled, they can mix together and measured by the mass spectrometer. This method enables simultaneous identification and protein quantification. The labels react with the N-terminus and reporter group is lost during the fragmentation, so this method can be used to determine the relative abundance of selected peptides of interest from 4 or 8 samples.

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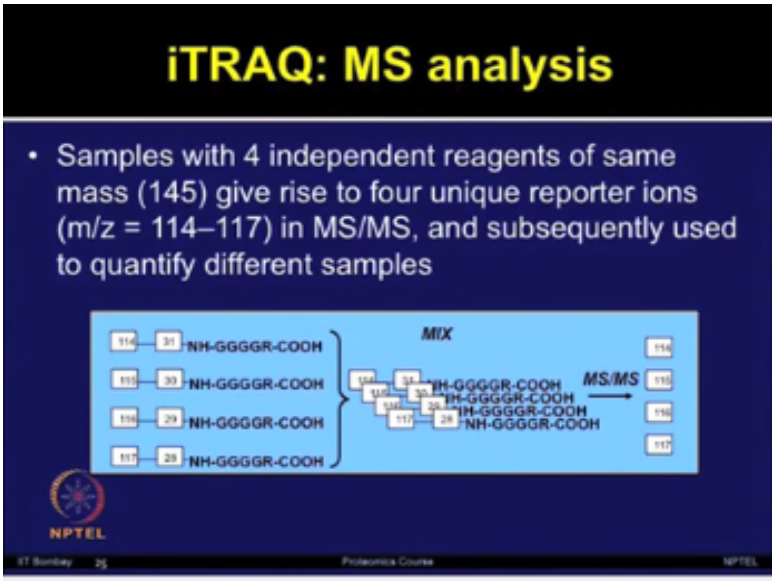

Therefore, high multiplexing can be obtained by using iTRAQ method. 4 independent reagents of same mass 145, can give rise to 4 unique reporter ions, m/z 114 to 117 in MS/MS analysis and subsequently these reporter ions can be used for quantification of different samples.

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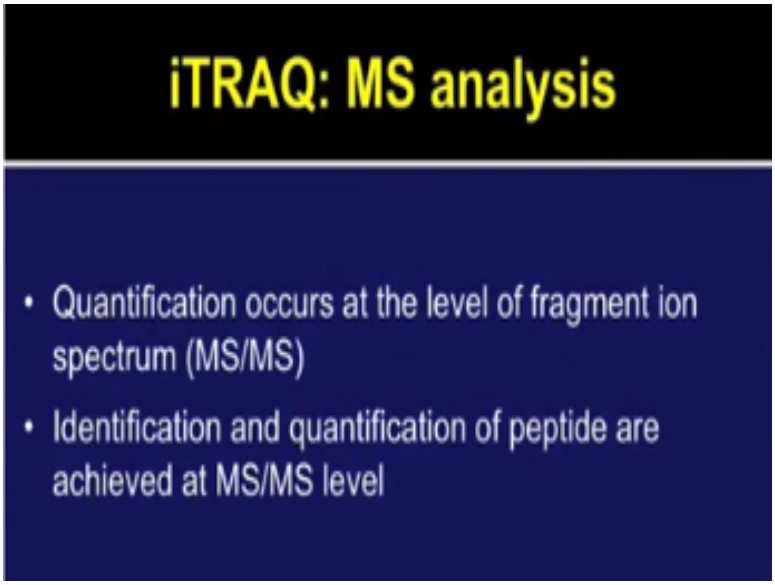

So, in iTRAQ the quantification occurs at the level of fragment ion, a spectrum at the MS/MS level, the peptides with the identical sequence but derivatives with different isobaric reagents are indistinguishable during the MS spectra alone, that is where only during the MS/MS fails, the quantification can happen. So, it shows that identical fragment ion series for the peptide derived fragment but it shows low mass reagent derived fragment ions that indicate that sample of origin and permit the quantitation.

So, identification and quantification of peptides are achieved during the MSS analysis. The iTRAQ enables the quantitative protein profiling of multiplex samples without making MS spectra complicated, it reduces the redundancy to selecting MS precursor ions for collision induced dissociation and enhances the efficiency for MS/MS analysis and peptide identification.

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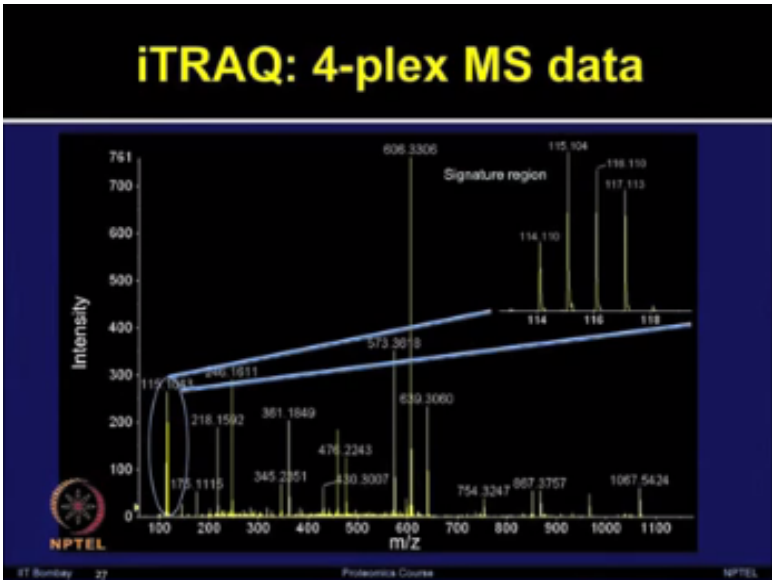

So, this is representative spectra for the 4 plex iTRAQ experiment, the MS data is shown and MS/MS spectrum is showing the reporter reagent, the signature of these 4, lets iTRAQ level be peptides 114, 115, 16 and 17.

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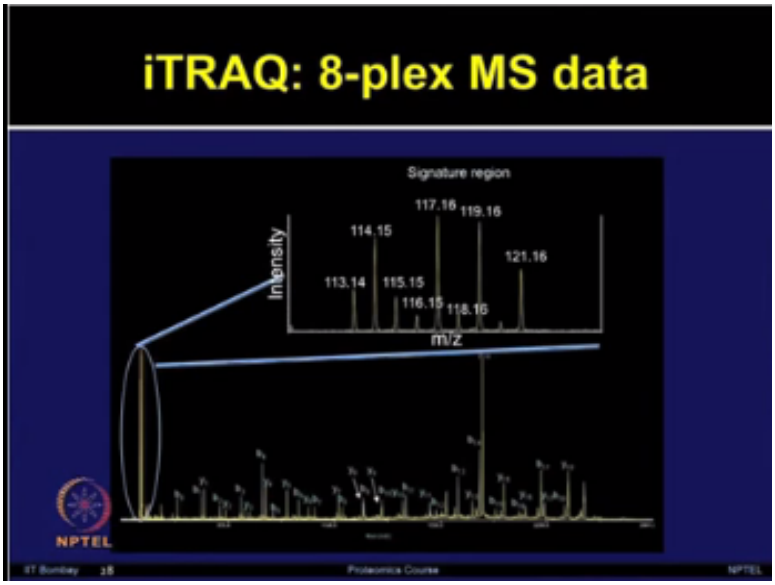

Now, we will have a look on the 8 plex MS data, so in MS/MS spectrum, now here we are showing the reporter reagent signature of an 8 plex iTRAQ reagent showing 113.14, 114.15,115.15, 116.15, 117.16, 118.16, 119.16 and 121.16 reporter ions. I will not describe you the overall iTRAQ technique by showing you an animation okay. In this animation, we will talk about iTRAQ technique.

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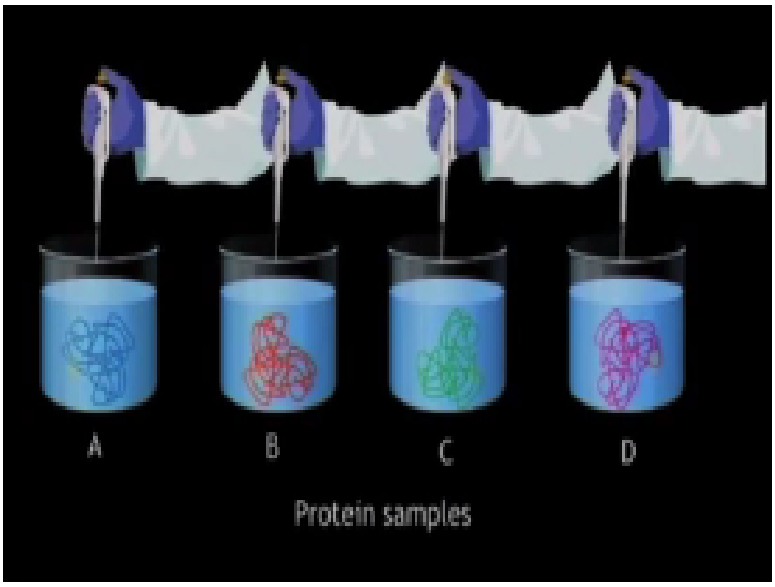

The protein samples to be analyzed our first digested with trypsin into some smaller peptide fragments.

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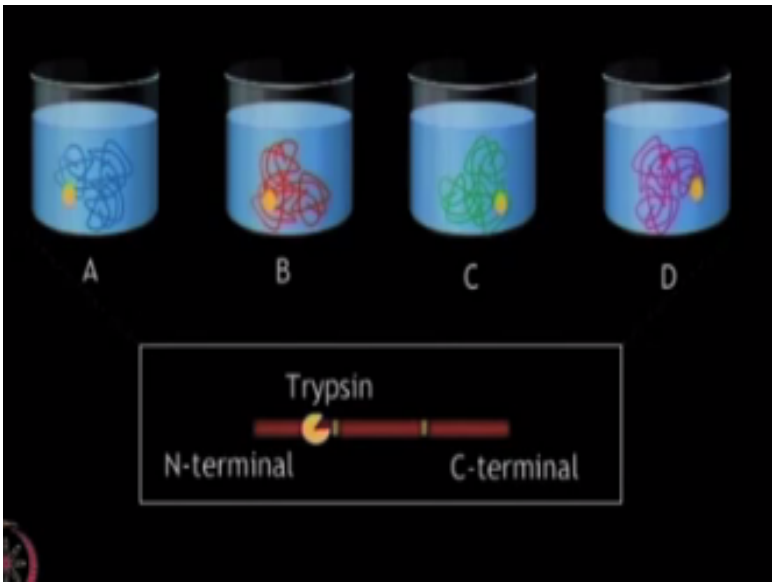

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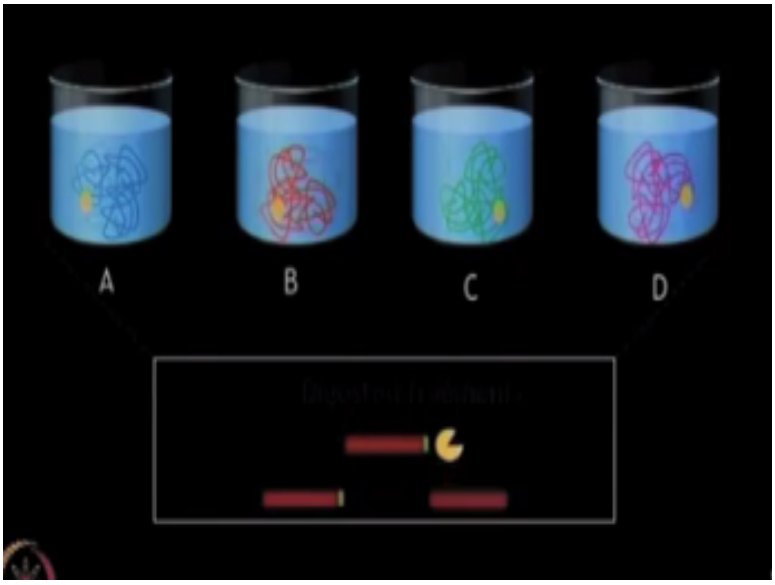

The trypsin cleaves the proteins at the C terminal of lysine and arginine residues unless they are followed by a prolene residue.

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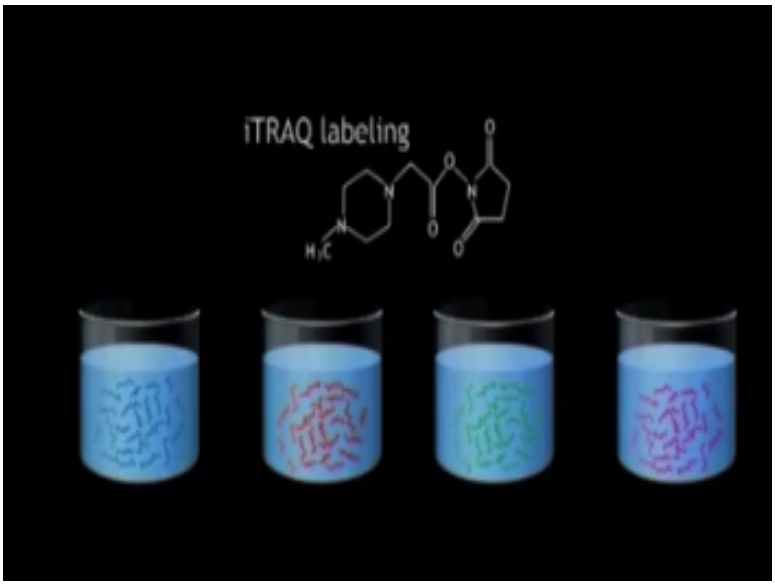

The iTRAQ reagent consists of a reporter group, a balanced portion and a peptide reactive group that interacts with the end terminus of the peptide or free amino group of lysine residues giving an overall mass of 145.

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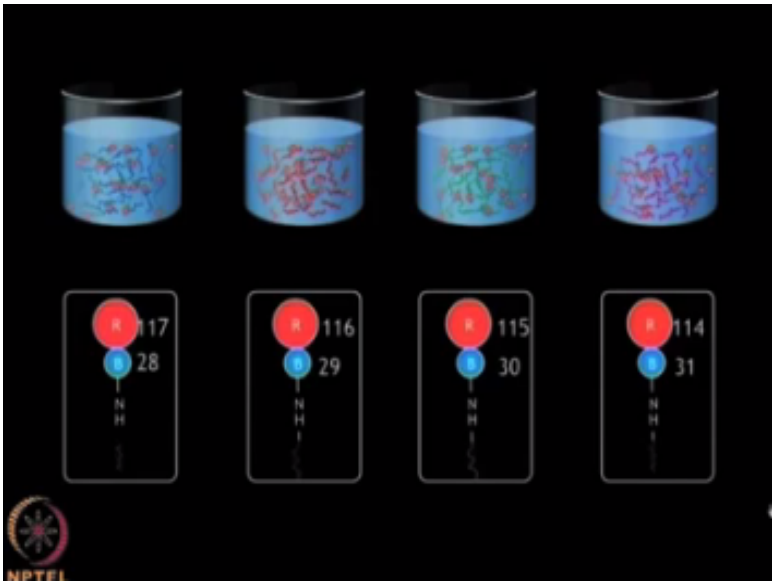

The reporter group used to label each peptide sample is unique and the mass are varying between the 114 to 117, thereby enabling the labeling and quantification of up to 4 samples simultaneously. As you can see in these animations and first one; the reporter group 117 and balancer group 28 in second sample reporter group 116, balancer group 29. In third sample, reporter group 115 and balancer group 30.

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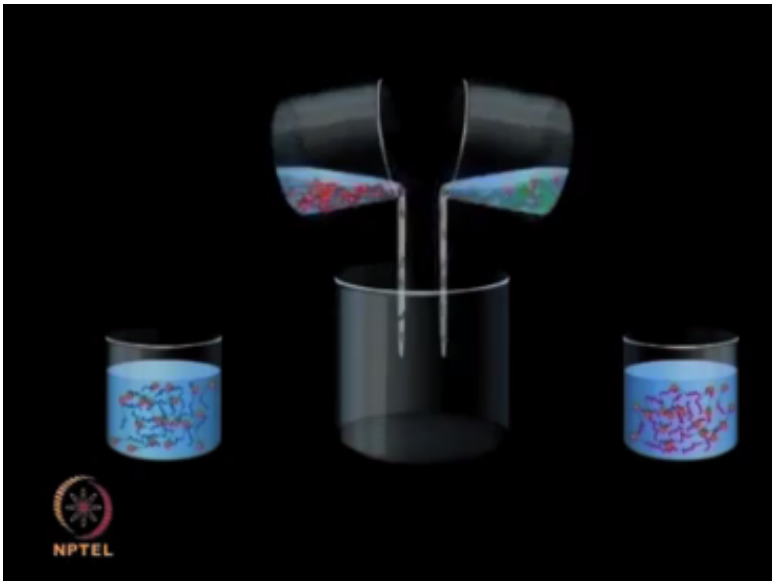

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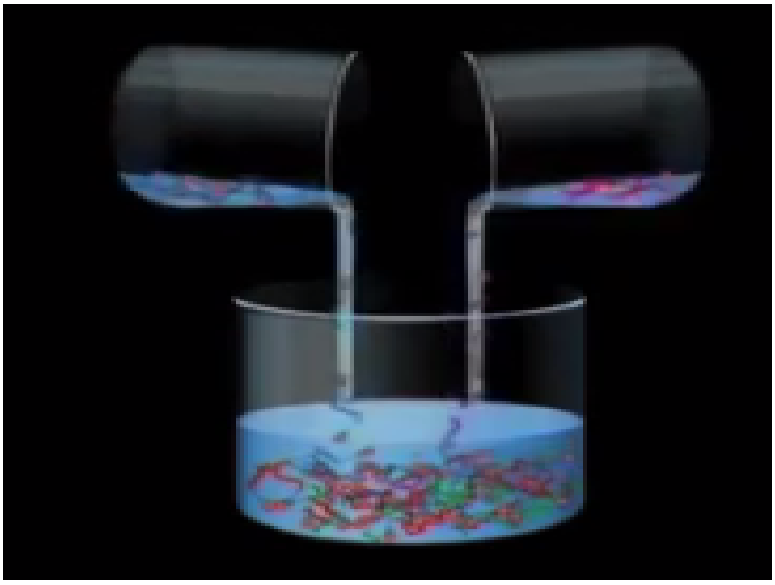

In fourth, the reporter group 114 and balancer group 31, overall 145 Dalton. This method has now further been improved to allow labeling of 8 sample simultaneously, although we are just showing here the 4 plex experiment.

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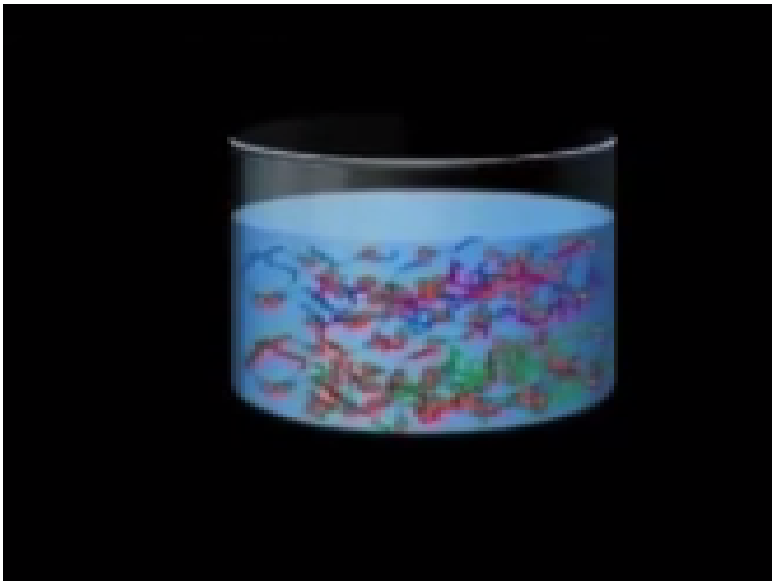

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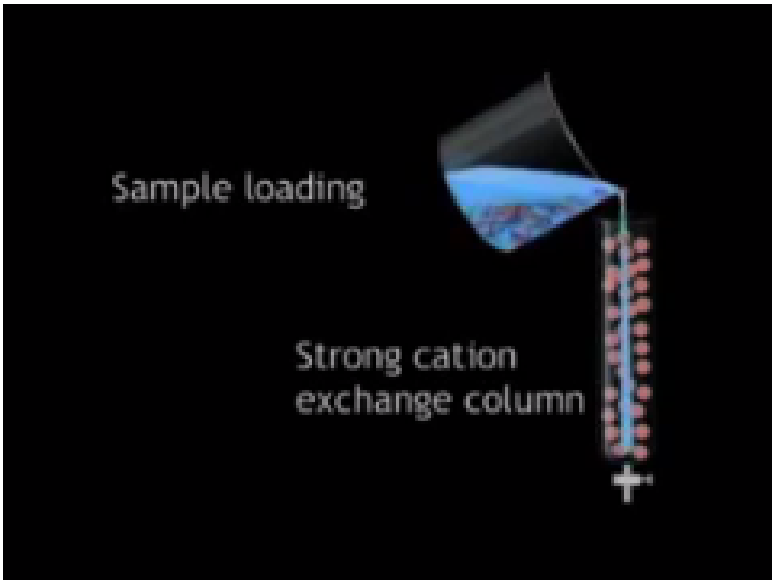

The label samples can be pooled together; the pool samples are purified on a strong cation exchange column to remove any excess unbound iTRAQ reagent.

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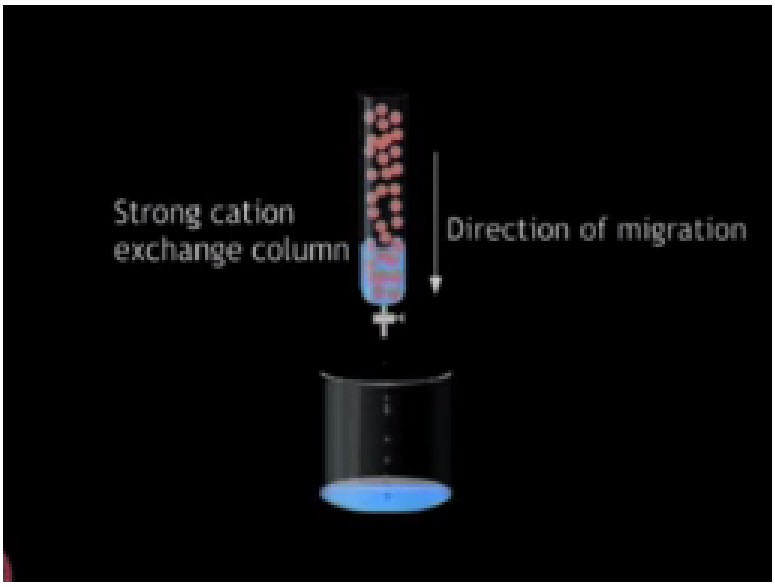

This facilitates the sample clean-up prior to further final separation and purification using reverse phase chromatography.

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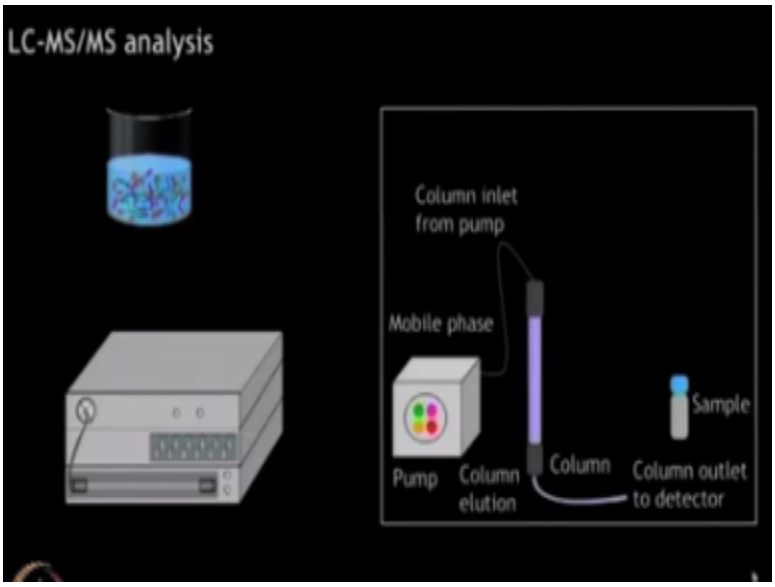

For the purification of strong cation exchange purified samples is carried out by reverse phase liquid chromatography, wherein the sample is passed through a column containing a packed stationary phase matrix that selectively adsorbs only certain analyte samples. The eluted fractions are further characterized by MS.

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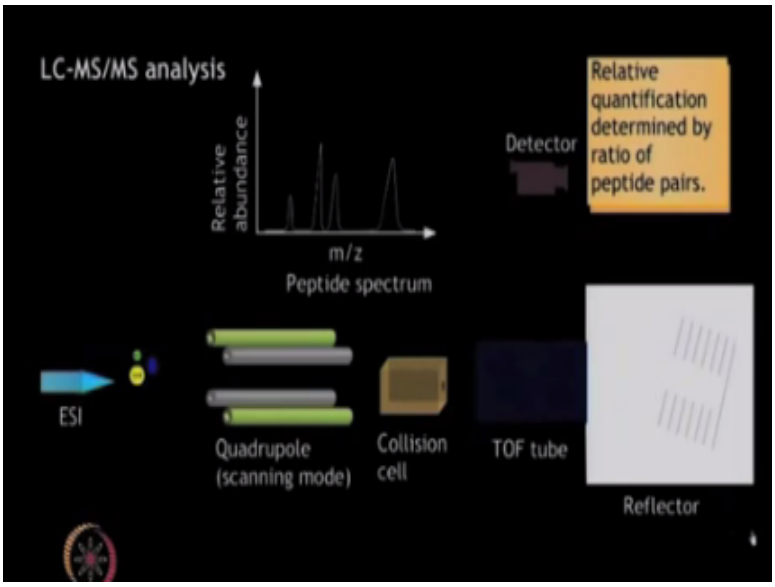

The purified label peptide fragments can be analyzed by MS/MS, the different masters of the reporter group allow the peptide fragments to be identified; the reporter group is lost during fragmentation step. The relative quantification of 4 samples or 8 samples can now be performed in this way.

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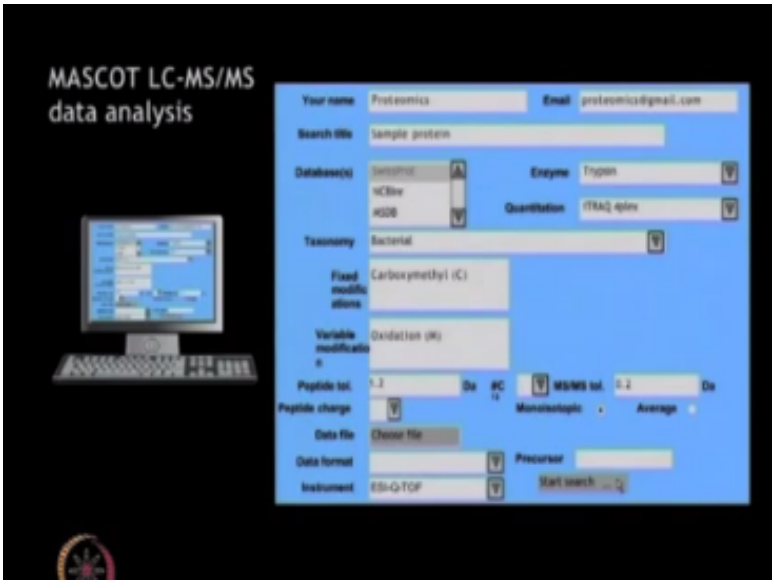

Once iTRAQ filament is completed, the data obtained from tandem mass spectrometry can be analyzed by using mascot and other software. The MS/MS data analysis requires input from the user regarding the experimental parameters such as enzyme cleavage, protein name, modifications, instrument used, peptide charge etc. The commonly used protein databases against which the MS information is processed to retrieve the sequence data include NCBI, MSDB and sis pro.

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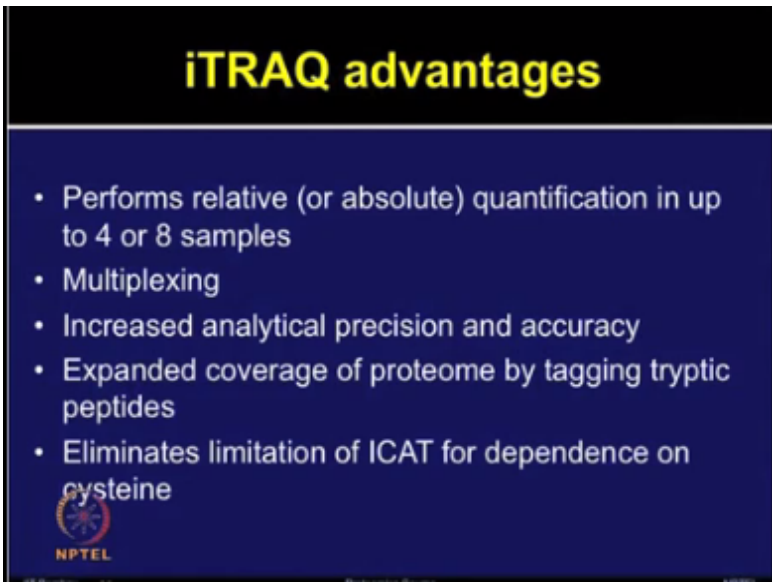

The data file generated from the MS is uploaded and a search can be carried out okay. So, I hope the animation was informative and now you are able to understand all the steps involved in the iTRAQ experiment. So, now let us talk about the advantages of using iTRAQ method. This method performs relative and absolute quantitation in up to 4 or 8 samples, it gives good multiplexing capability.

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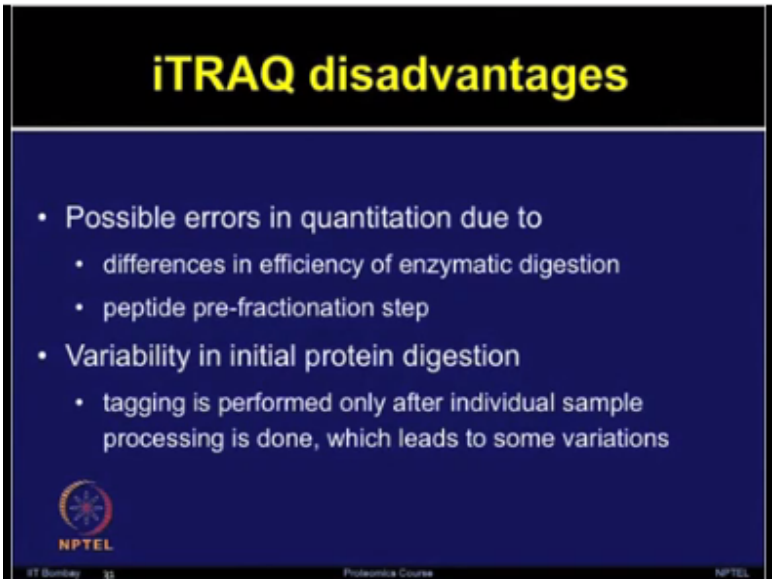

It increases the analytical precision and accuracy, the expanded coverage of proteome can be obtained by iTRAQ method by tagging the tryptic peptides, it eliminates the limitation of the previously discussed method the ICAT for dependence on cysteine residues but this method has several disadvantages as well. First of all, there is possibility of errors in the quantification due to the difference in the efficiency of enzymatic digestion.

Because we are performing that step separately for 4 samples, if you remember we talked about sample A, B, C and D, each one of those we treated separately and if during the tryptic digestion, the efficiency was not exact for the digestion process maybe that could lead to some difference although, if you start with the same enzyme and if you have done the quantification for the proteins properly.

Ideally, there should not matter but there is some possibility that some errors could be coming from that efficiency of enzymatic digestion. The peptides pre fractionation step could be another possible way of introducing some variations, the variability in the initial protein digestion and then tagging is performed only after the individual sample processing is done, so if we can do the multiplexing in some way that peptic digestion cannot be performed separately that can reduce the overall chances of error.

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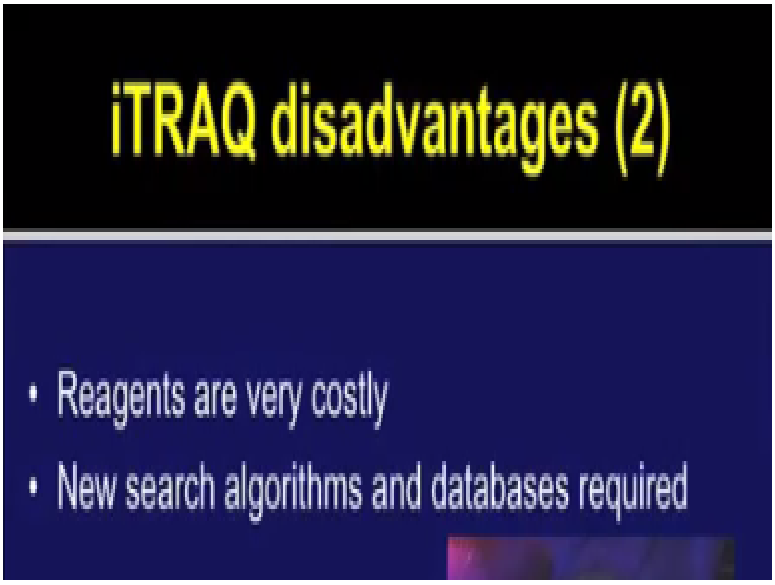

But that will actually not be possible because we need to label each samples separately with the iTRAQ reagents. So, these are still some of the possible errors and disadvantages of iTRAQ method. Now, these reagents are quite costly, so that is also a limiting factor for many labs, do not able to use that and there are various search algorithm and databases are required obviously with more and more new studies are happening.

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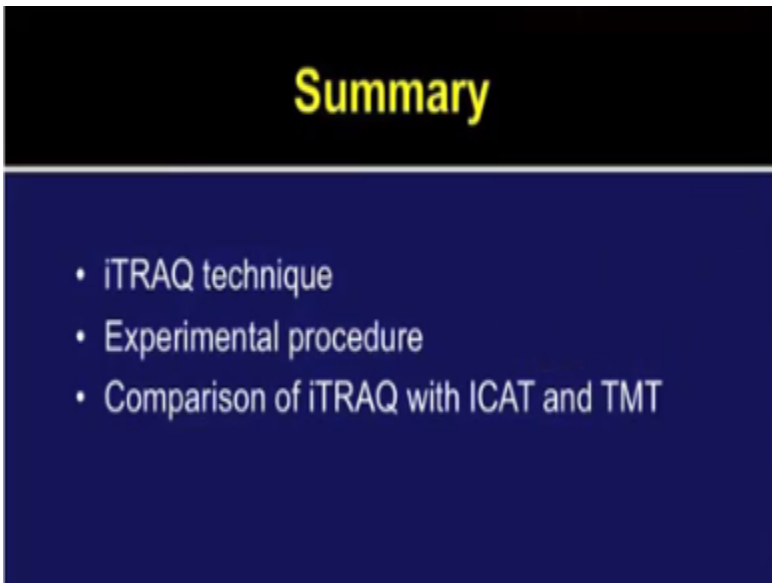

So, now we have better software and better information available for performing these experiments but it still that is one of the limitations. So, overall in today's lecture, we talked about iTRAQ technique, we compared iTRAQ with ICAT and TMT and during the discussion, we also look in much more detail about how to perform the iTRAQ experiment, so as you know with STD advances in the mass spectrometry, the quantitative proteomics has progressed dramatically in the past few years.

They are first to analyze proteome of many species both qualitative and quantitative have generated an abundance of data in a variety of balance and samples from bacteria to human. The chemical labeling using isobaric tags for relative and absolute quantification iTRAQ or tandem mass tag, TMT reagent, the quantification is based on the extraction of reporter ions from tandem mass spectrometry, MS/MS spectra.

These methods can be used on all kinds of balanced samples and provide high level of reliability for the contact of data. So, we will continue our discussion about quantitative proteomic techniques in the next lecture on in vivo based stable isotope labeling methods and then we will try to compare the in vitro and in vivo based labeling method. Thank you.